

## Defining the Limbal Stem Cell Niche

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### Abstract

The cornea forms the front transparent cover of the eye which is reputed to be maintained by a population of adult stem cells located at the junction of the cornea and the white sclera of the eye. This area is known as the limbus and the stem cells as limbal stem cells. The desire to describe the exact location of the stem cells at the limbus has led to several descriptions of anatomical features that could provide the niche environment for these cells. Our laboratory, acting upon evidence that the limbus is not the sole location of adult stem cells in the cornea, analysed whether the limbal stem cells could exist outside of the limbal environment and whether they would still retain the properties required to maintain the corneal surface.

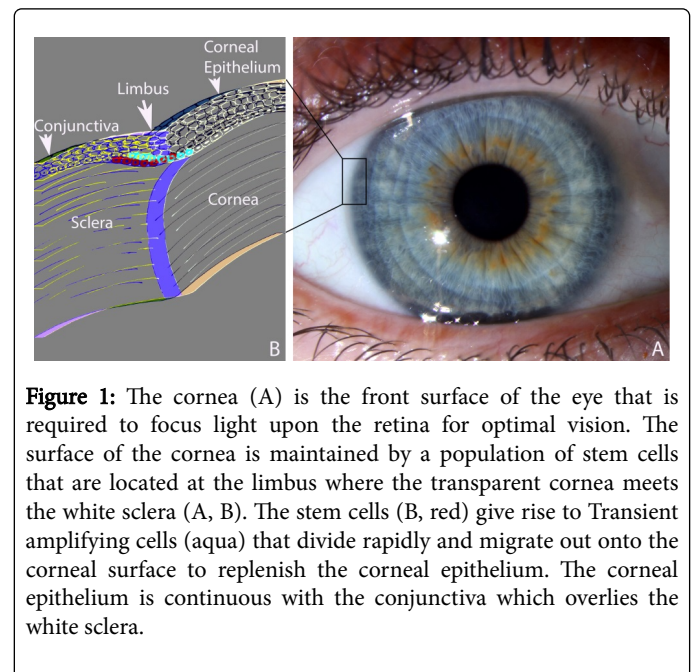
### Keywords:

Cornea; Limbal stem cell; Conjunctival tissue

### Introduction

Maintenance of the transparent front surface of the eye is imperative to light entry and refraction onto the correct position on the retina. The cornea (the transparent front surface of the eye) (Figure 1A) together with the iris and pupil form the anterior chamber, with the periphery of the anterior tissues meeting the white opaque sclera and its overlying transparent conjunctiva at the limbus. The anterior-most ocular surface is composed of corneal and conjunctival epithelia with the limbus at the transition zone between the two (Figure 1B). This limbal zone exhibits multiple anatomically unique features and is the area in which the major population of adult stem cells that replenish the corneal surface are thought to reside.

All stem cells are thought to require a niche within which their stemness can be preserved. A stem cell niche is an anatomically defined area that is thought to provide a variety of intrinsic and extrinsic factors such as the physical protection, survival factors and cytokines and deemed essential to the maintenance of a stem cell population while preventing entry into differentiation [1,2]. Over the past decade, much progress has been made in characterising the putative niche in the limbus.



**Figure 1:** The cornea (A) is the front surface of the eye that is required to focus light upon the retina for optimal vision. The surface of the cornea is maintained by a population of stem cells that are located at the limbus where the transparent cornea meets the white sclera (A, B). The stem cells (B, red) give rise to Transient amplifying cells (aqua) that divide rapidly and migrate out onto the corneal surface to replenish the corneal epithelium. The corneal epithelium is continuous with the conjunctiva which overlies the white sclera.

This research has focused on the identification of a niche environment (considered essential to preserve stemness) for the limbal stem cell and has resulted in several hypothetical niches being proposed for the peripheral cornea; palisades of Vogt [3], limbal epithelial crypts [4] and focal stromal projections [5]. These structures are characterised by their close proximity to blood vessels and distinct matrix formations providing undulating structures that increases surface area. These characteristics are thought to facilitate adhesion, physical protection and provide growth factors and nutrients [2]. The importance of the niche environment has led to the contention that stem cells cannot be studied in isolation, and that *in vitro* expansion systems must mimic components of the *in vivo* niche [6]. We aimed to investigate which characteristics of the niche ie. anatomical position,

proximity to vessels, proximity to growth factors and matrix specialisations were actually important in defining limbal stem cell function.

Many signalling pathway molecules have been linked with the limbal niche and the cells therein. The sonic hedgehog, Wnt/ $\beta$ -catenin, TGF- $\beta$  and Notch signalling pathways have all being implicated in niche control of stem cells outside of the eye, however less is known of their potential roles in the limbal niche. Further analyses centred upon the signalling pathways that control cell cycling and self-renewal regulation of limbal stem cells and progenitor cells including WNT and Notch. These results have shown that Wnt/ $\beta$ -catenin signaling increases the proliferation and colony-forming efficiency of human limbal stem cells while preserving the positive and negative limbal stem cell markers. Whilst, on the other hand, the high levels of notch at the limbus correlates with decreased cell proliferation [7].

Within these niches, the limbal stem cells divide asymmetrically to provide one stem cell daughter and one transient amplifying cell. The transient amplifying cell then divides multiple times to provide many daughter cells that travel centripetally over the corneal surface and mature into differentiated epithelial cells that move anteriorly towards the outer layers of the 5-7 layered epithelium, before being sloughed off by desquamation to provide a constantly renewing corneal epithelium.

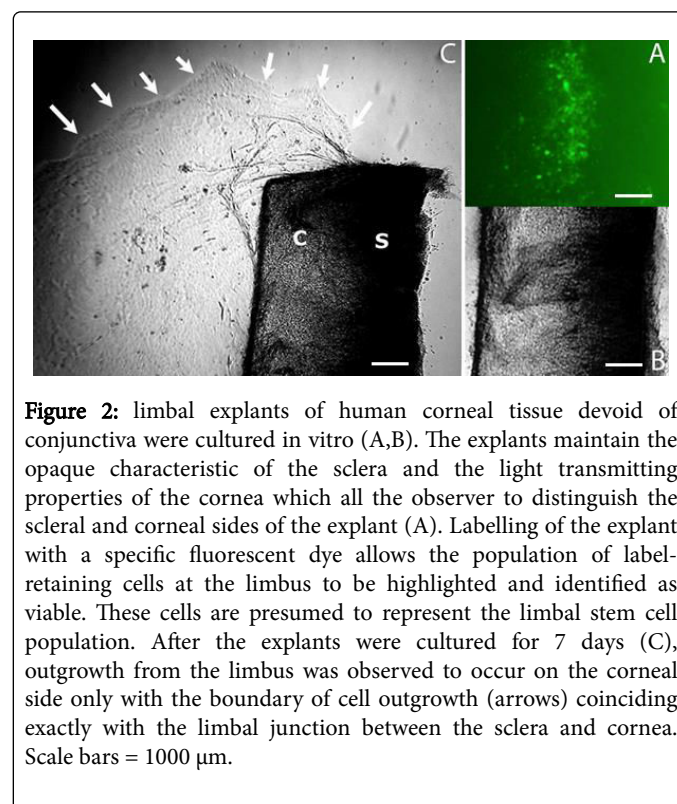
Furthermore, the limbus is also thought to be the barrier that stops the conjunctiva from over-running the corneal surface. Thus the replication of the limbal stem and amplifying cell populations provide a physical presence which can keep the conjunctiva at bay. This theory would predict that removal of the stem cells at the limbus would remove the constantly renewing population of cells that physically restrain the conjunctiva and thus would allow the advance of this tissue over the corneal surface. This condition exists and is called limbal stem cell deficiency and can be caused by various conditions or environmental factors including UV exposure and chemical burns. Occurrence of limbal stem cell deficiency does indeed allow conjunctivalisation of the cornea and thus seemingly supports the existence of the limbus as a physical border between two proliferating populations. However this relationship is not a straightforward one, since central corneal epithelial populations have been described as maintaining the corneal surface and resisting further ingress by the conjunctiva in the absence of limbal input due to full 360° limbal stem cell deficiency for up to 12 years [8].

Several elegant studies have shown the centripetal movement of the epithelial cells from the limbal area towards the centre of the cornea [9]. However the question remains as to whether this is purely a product of a bullying conjunctival population that allows the daughter cells of the limbal stem cells to move in one direction only and that is towards the centre of the cornea. Such a system would provide a simple mechanism of corneal renewal that relies on pressure from the outside rather than any inherent characteristics of the limbal stem cell population.

Our laboratory has conducted several studies that look at the characteristics of this limbal population and the cell properties and cell-cell interactions that contribute to the unique properties of this anatomical feature.

Firstly, we wanted to investigate how the limbal cell population would react if it found itself suddenly freed of the pressure from the conjunctiva. Would the daughter cells of the limbal population be able

to move centrifugally out over the sclera if the conjunctiva were to be removed. This would determine whether the daughters of the limbal stem cells preferentially move centripetally onto the corneal surface because of the wall of conjunctiva blocking their movement in the other direction? Or is the centripetal movement of cells from the limbus to cover the corneal surface driven by the molecular and cellular characteristics of the limbus governed independently of the conjunctiva? We took limbal explants from which all conjunctival tissue had been removed and plated them on tissue culture surfaces that promoted cell growth and migration (unpublished data). The explants exhibited light transmitting properties that enabled us to distinguish the clear corneal tissue from the more opaque sclera by light microscopy. Labelling with specific dyes enabled us to identify a population of dye retaining cells that located to the limbal region and likely defined the limbal stem cell population of slowly cycling cells. Over a period of 7 days, we monitored cell migration from these explants and found that the growth from the limbus was directed towards what would have been the corneal surface and no growth towards the scleral side had occurred. This indicated to us that the limbal stem cells acted independently of the conjunctiva (as no conjunctiva was now present) and the centripetal migration of cells from the limbus towards the centre of the corneal surface was determined by the limbal populations themselves (Figure 2).

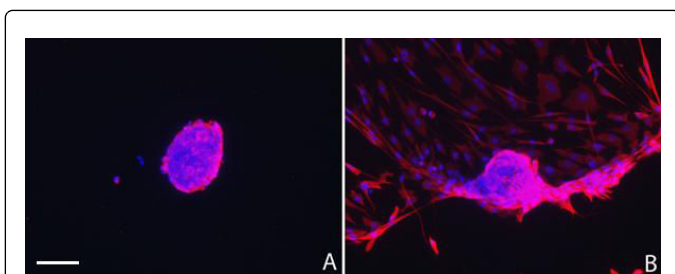


**Figure 2:** limbal explants of human corneal tissue devoid of conjunctiva were cultured in vitro (A,B). The explants maintain the opaque characteristic of the sclera and the light transmitting properties of the cornea which all the observer to distinguish the scleral and corneal sides of the explant (A). Labelling of the explant with a specific fluorescent dye allows the population of label-retaining cells at the limbus to be highlighted and identified as viable. These cells are presumed to represent the limbal stem cell population. After the explants were cultured for 7 days (C), outgrowth from the limbus was observed to occur on the corneal side only with the boundary of cell outgrowth (arrows) coinciding exactly with the limbal junction between the sclera and cornea. Scale bars = 1000  $\mu$ m.

This led us on to investigations that resulted in a series of papers looking at cells isolated from the peripheral cornea and limbus and trying to determine the properties within those cells that determined the fate of daughters deriving from them. One method of isolating cells with proliferative potential is a sphere-forming assay where the tissue extracts are cultured in mitogen-driven serum-free medium on non-adhesive substrate [10].

We used methods to derive cells from human limbal tissue which in culture allowed the extracted cells to aggregate and form spheres of cells which survived many months in culture and selected for the least differentiated cells isolated from that tissue. Spheres stained positively for stem cell markers  $\Delta$ NP63 $\alpha$  ( $\Delta$ N isoform of P63 $\alpha$ ), ABCG2 (ATP-binding cassette sub-family G member 2) and ABCB5 (ATP-binding cassette sub-family B member 5) (considered to be the best current markers for limbal stem cells, but also probably define the first daughter transient amplifying cells within the limbal environment) [11,12] as well as the basal limbal marker and putative niche marker, notch 1. In addition, spheres also stained positively for markers of corneal cells, vimentin, keratin 3, keratocan and laminin, indicating a heterogeneous mix of stromal and epithelial-origin cells. We then tested what the migration characteristics of these spheres on a collagen substrate in the absence of a limbus would be.

This study [13] found that these peripheral corneal spheres showed spontaneous polarised outgrowth (Figure 3).



**Figure 3:** A sphere of cells isolated from the peripheral cornea is labelled for tubulin in red and DAPI in blue showing the highly defined nature of these spheres (A). When the spheres are allowed to migrate on a collagen surface exhibit polar outgrowth and directed cell migration (B). Scale bar = 100  $\mu$ m.

Spheres occasionally also showed polarised outgrowth followed by collective migration with discrete morphological changes to form leading and trailing compartments and placement of spheres in close proximity led to production of a cell exclusion area adjacent to spheres. This clearly showed that peripheral corneal cell spheres are dynamic entities capable of developing polarity and modifying migration in response to their environment but remarkably independently of the limbal environment.

We have previously performed experiments on human corneas where we lasered donut ablations in the corneal epithelium and were able to show that central corneal epithelium was capable of cell division and cell migration towards the limbus [14]. Even if the limbal cells themselves were abated, cells from the central cornea would move towards the limbal area to fill the void that had been created. Taking this data in conjunction with the data above, we then decided to examine the migration properties of the peripheral corneal spheres when transplanted back onto a denuded corneal stroma and adjacent limbus [15].

After implantation of peripheral corneal spheres into decellularized corneoscleral tissue, we observed polarised and radially orientated cell migration with cell proliferation was observed. Cells migrated out from the spheres and repopulated the entire corneal surface over 14 days. However, the manner of ocular surface repopulation over the corneal surface contrasted with observations over the sclera,

suggesting that the regional difference in substrate composition exerts an effect on cell migration. Cells from the sphere which completely repopulated corneal tissue demonstrated a preferential migration in the corneal direction in comparison with the scleral direction with cells migrating much greater distances towards the central cornea from the site of implant. A close examination of the orientation demonstrated by migrated cells revealed the regular, parallel arrangement of cells aligned with their long axes oriented toward the central cornea, while cells which repopulated the limbal region aligned circumferentially and the limited number of cells which migrated onto the scleral region appeared to be in random orientation.

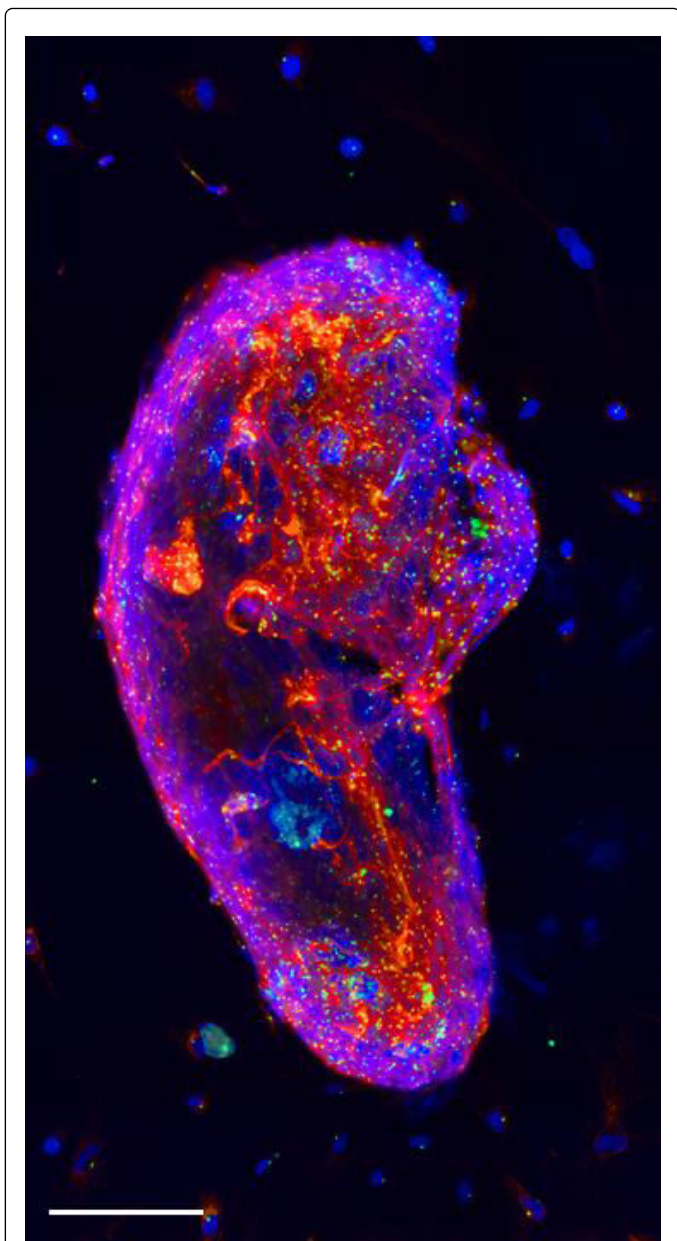
This clearly showed that sphere cells migrate to repopulate the denuded cornea tissue but upon reaching the limbus their migration patterns become re-aligned and they show a distinct lack of preference for the scleral surface.

At this point we re-examined data again and it was noticeable that cell aggregation from our peripheral corneal isolates tended to nucleate around extra cellular matrix debris as we always observed a dense auto fluorescent core at the centre of our sphere aggregates. Then as the spheres mature and are shown collagen based substrates the spheres show distinct polarity and cells migrate preferentially out from one hemisphere. Moreover the cells upon the long axis of migration seem to form a ridge like structure that resembles the cell alignment patterns that we observe when cells interacted with denuded limbal tissue (Figure 1). When several spheres appear in proximity the orthogonal ridges forming from one sphere show a tendency to join up with the cell spur growing out from the second sphere and the now joined spheres coordinate their growth planes and the cell exclusion zones accordingly.

Some insights into the polarised nature of these spheres of stem cells are evident when we examine the spheres prior to migration (Figure 4). Cells within spheres show highly polarised patterns of organisation ready to migrate in preordained direction even after a short exposure to a suitable migration surface. We used pericentrin staining to define the microtubule organising centre of individual cells within the sphere. It was obvious that three centres of cells were nucleating and would form the epicentres of cell migration upon stimulation. The position of these 3 apices would lead to 180 $^{\circ}$  of cell migration.

Thus in conclusion we hypothesise that the stem cells of the cornea act independently of their limbal location and it is the stem cells themselves that establish the niche rather than any anatomical feature found in the limbal region. It is the inherent polarised and directed migration properties of stem cell populations together with a preference for migrating onto the corneal surface that determine the centripetal movement of cells rather than the pressure exerted from the conjunctival tissue. These properties are independent of proximity to blood vessels or any structures found at the limbus and indeed the stem cell aggregations show the ability to reform limbal like structures rather than being dependent upon proximity to the limbus for their function.





**Figure 4:** A sphere of corneal stem cells stained for DAPI (blue) tubulin (red) and pericentrin (which localises as green dots which highlight the tiny MTOC located within each cell) highlight the polar arrangement of cells within the sphere. Pericentrin staining highlights the accumulation of 3 distinct centres of cell aggregation which would lead to the migration patterns observed in Figure 3. Scale bar = 100  $\mu$ m.

These findings have important consequences for the potential to use these stem cells spheres as therapeutic treatments to re-establish a limbal stem cell population following limbal stem cell deficiency even in cases where the limbus itself is significantly traumatised. The evidence shows that these spheres maintain the intrinsic properties of stem cells show the correct polarity and are capable of the directed cell migration necessary to continually restore the corneal surface and are even capable of rebuilding a limbal like structure.

The only question remaining is whether these stem cell spheres retain the ability to respond to trauma by rapid proliferation and migration to repair the injury [16]. This ability is a characteristic of the native stem cell population and would be required to be retained if the stem cell spheres were used to rebuild a corneal limbus and then protect it against a secondary insult such as trauma. We analysed the ability of the stem cell spheres to react appropriately to a traumatic insult. We conducted direct compression injury and remote scratch injury experiments on the cell spheres to gauge their wound healing capacity. Measures of proliferation, differentiation, and migration were assessed by immune histochemical detection of EdU (5-ethynyl-2'-deoxyuridine) incorporation,  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) expression and confocal image analysis, respectively. Both types of injury drew responses from the spheres indicating appropriate wound-healing processes. Direct wounding induced a rapid, but transient increase in expression of  $\alpha$ -SMA, a marker of corneal myofibroblasts (the cell produced in response to corneal injury), followed by a proliferative and increasing migratory response. The spheres were observed to respond to remote injury as entire units, with no directional response seen for targeted repair over the scratch injury area. These results give strength to the future use of these peripheral corneal spheres as transplantable units for the regeneration of corneal tissue.

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